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**Local lung responses following local lung challenge with recombinant
lungworm antigen in systemically sensitised sheep**

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Abstract

Background: Chronic mast cell-mediated inflammation may contribute significantly towards the extensive tissue remodelling that is a feature of lungworm infection in ruminants. Understanding the factors that control tissue remodelling is a necessary step toward effective management and treatment of conditions that feature such pathology. **Objective:** We sought to define in a novel ovine model system, the cellular, immune and mast cell phenotypic events that occur following local lung challenge with a recombinant protein antigen, DvA-1, derived from the ruminant lungworm nematode, *Dictyocaulus viviparus*. **Methods:** Two spatially disparate lung segments in systemically sensitised sheep were challenged on three occasions with DvA-1 (3xDVA) and two further segments were challenged with saline (3xSAL). Two months after the third challenge, one of the two segments previously repeatedly challenged with DvA-1 was challenged again with DvA-1 (3xDVA:DVA) whilst the other was challenged with saline (3xDVA:SAL). A similar protocol was followed with the saline challenged segments (3xSAL:SAL & 3xSAL:DVA). Bronchoalveolar lavage fluid (BALF)(n=16) and tissue (n=3) were collected after the last challenge. **Results:** Cellular changes 24 h after the fourth challenge were characterised by an increase in the absolute numbers of neutrophils and eosinophils in BALF from 3xDVA:DVA and 3xSAL:DVA segments. Local antibody production was implied through increased levels of antibody in both 3xDVA:DVA and 3xDVA:SAL segments, with the latter being unaffected by inflammation. Levels of active transforming growth factor beta-1 (TGF- β 1) were significantly increased in 3xDVA:SAL segments and a trend towards an

increase was apparent in 3xDVA:DVA segments. Total TGF- β 1 levels were significantly correlated with eosinophil counts in all except the 3xDVA:SAL segments. Such changes in the bronchoalveolar space were complemented by increased ratios of sheep mast cell proteinase-1 expressing cells and tryptase expressing cells, to toluidine blue positive cells in airways from 3xDVA:DVA segments. **Conclusion:** Mast cell phenotypic events occurring as a consequence of antigen challenge were limited to segments in which changes in BALF were characterised by neutrophil influx *and* increased local antibody production.

Introduction

The pathology of patent lungworm (*Dictyocaulus spp.*) infection in ruminants is characterised by chronic catarrhal bronchitis and bronchiolitis, bronchial epithelial and mucous cell hyperplasia, increased peribronchiolar fibrous tissue and smooth muscle, and prominent eosinophil and mast cell infiltrates [1] [D.Collie pers. observation]. Understanding the factors that control the tissue remodelling processes that underlie the pathology would be of some relevance to human diseases such as asthma where pronounced airway wall remodelling occurs [2].

Mast cells are likely to play a fundamental role during tissue remodelling processes. In addition to the expression of matrix components (type VIII collagen alpha-1 chain) [3], mast cells contain an abundance of mediators (VEGF, FGF-2, PDGF, TGF-beta, NGF, IL-4, IL-8) that are at least potentially directly linked to neoangiogenesis, fibrinogenesis or re-epithelization processes. Central among such mediators is transforming growth factor- β 1 (TGF- β 1). This growth factor promotes ECM deposition by stimulating different collagen, elastin, fibronectin and proteoglycan genes to produce these ECM components [4-6]. TGF- β 1 down-regulates proteinase genes and up-regulates proteinase inhibitor genes [7,8] and is also a potent immunosuppressive agent [9] and a potent chemoattractant under certain circumstances [10].

In addition, mast cells, through the release of TNF- α and other cytokines, can importantly influence the recruitment and/or function of additional effector cells such as eosinophils, neutrophils and lymphocytes [11].

Mast cells are a phenotypically heterogeneous population with respect to tissue site, fixation properties, histochemical staining, biochemistry and functional activity. This heterogeneity is particularly reflected in the expression of mast cell granule proteases. In man, mast cells comprise a population that contains tryptase and chymase (MCTC) and one that contains only tryptase (MCT). The MCTC is found predominantly in skin and at subepithelial locations in the bronchial, nasal, and gastrointestinal mucosa, whereas the MCT is located predominantly in alveolar walls, gastrointestinal epithelium, and in the airway epithelium of patients with allergic disease.

Similarly, sheep demonstrate mast cell heterogeneity. Using a polyclonal anti-human tryptase antibody and a monoclonal antibody against sheep mast cell protease-I (SMCP-I), a chymase originally isolated from gastric mast cells, it is possible to demonstrate variation in immunoreactivity depending on the specific anatomical location. For example, SMCP-I is expressed in mast cells in the mucosa of the gut but not in the submucosa or adjacent muscularis [12]. Interestingly, in the lung, available evidence points to minimal SMCP and maximal tryptase immunoreactivity in mast cell populations surrounding the airways in normal sheep [12].

Dynamic flux in the relative proportions of phenotypically distinct mast cell populations may have important bearing on lung allergic responses. Indeed, in murine rodents, the β -chymases, mouse mast cell protease-1 (mMCP-1) and rat mast cell protease -II (rMCP-II), which characterise mucosal mast cells in these species, are secreted systemically and into the gut lumen during

gastrointestinal allergic responses [13,14]. Further, transforming growth factor beta-1 (TGF- β 1) has recently been implicated as a potential influence on such expression in the mucosal environment [15].

In an effort to elucidate the mechanisms underlying mast cell mediated events in ovine allergic pulmonary disease we have developed a novel model based on systemic sensitisation with a recombinant antigen, DvA-1, from the bovine lungworm *D. viviparus*. DvA-1 is a retinoid-binding polyprotein and has similar sequence and deduced structural similarities to the ABA-1 allergen found in the respective human and porcine gastro-intestinal nematodes *Ascaris lumbricoides* and *Ascaris suum* [16,17]. We report here on the inflammatory and immune events that occur following local lung challenge with the same antigen.

Materials and Methods

Animals: Sixteen crossbred sheep (Shetland x and Merino x; 8 F and 8 MN) (bodyweight: 26 - 49 kg) were used in this study. Animals were treated with anthelmintic prior to entry into the study and were adjudged to be free from significant pulmonary disease on the basis of clinical examination.

Production of recombinant DvA-1 polypeptide: Production of recombinant polypeptide was essentially as previously described [17]. Briefly, DNA encoding DvA-1 was amplified from plasmid pET 15b (Novagen, Madison, WI, USA) as a His-tag fusion protein and expressed in BL21 *E. coli*. Endotoxin levels, estimated by a kinetic turbidimetric limulus amoebocyte lysate (LAL) assay, did not exceed 0.8 EU (endotoxin units)/ml of DvA-1 stock (prepared to a concentration of 2mg/ml).

Sensitisation with DvA-1: Sheep were sensitised with DvA-1 adsorbed to $\text{AL}(\text{OH})_3$ gel (Alhydrogel, 20mg/ml; Accurate Chemical & Scientific Corporation, Westbury, NY) using 50 μg DvA-1/500 μl of $\text{AL}(\text{OH})_3$ in sterile water to 1ml, given intramuscularly on three occasions at biweekly intervals.

Challenge: DvA-1 antigen was dissolved in 1ml saline to a final concentration of 400 $\mu\text{g}/\text{ml}$. A nebulisation catheter (Trudell Medical International; London, Canada) placed in the biopsy channel of a fiberoptic bronchoscope was used to deliver the aerosolised solution to defined lung segments over a period of approximately 60 seconds.

Anaesthesia and ventilation: Food was withheld for 12 hours prior to anaesthesia which was achieved by intravenous administration of a single bolus of thiopentone sodium (Intraval sodium; Rhône Mérieux Ltd.) at a dose rate of 20 mg/kg bodyweight. Thereafter sheep were intubated and anaesthesia maintained using gaseous halothane (2-3 per cent) in oxygen and nitrous oxide. The sheep were placed in sternal recumbency in a large plexiglass whole body respirator (internal volume: 143 litres). The proximal end of the endotracheal tube was connected to the anaesthetic circuit through a connector in the wall of the box. Pressure in the box was varied by appropriate connection to a large bellows pump (Cuirass; Cape Warwick, Warwick, U.K.) which induced a sinusoidal tidal respiratory pattern, the rate and magnitude of which could be controlled by adjustment of the pump itself. The magnitude of pressure fluctuations were adjusted to maintain a tidal volume of 10ml/kg bodyweight.

Respiratory rate was adjusted to maintain end-tidal CO₂ measurements in the range 4.5-5.5% (Oxicap Monitor Model 4700; Ohmeda, Louisville, CO, USA).

Bronchoalveolar lavage: A flexible fibre optic bronchoscope (5.3mm OD)(Model FG-16X; Pentax U.K. Ltd.) was advanced and wedged in selected segmental bronchi. The subtended segments were lavaged by installation and withdrawal of two, 20ml aliquots of normal saline (0.9% NaCl solution). BALF was immediately placed on ice until subsequent analysis.

Sample handling: Lavage fluid from each lung lobe was separated into supernatant and cells by centrifugation at 400g for 7min and the resultant cell pellets were resuspended in phosphate buffered saline (PBS) before differential cytology was determined. Supernatants were recentrifuged at 1,000 x g at 4°C for 20 min. The samples were stored at -70°C until analysis of immunoglobulin and TGF-β1 levels.

Differential cytology: Cells were counted using a Neubauer haemocytometer and values expressed per millilitre BALF. Cytocentrifuge slides were prepared and stained using Leishmann's stain for differential counts on 400 cells. Cells were classified as neutrophils, macrophages, eosinophils, lymphocytes or mast cells according to standard morphological criteria. Cells not classified in the aforementioned groups were recorded as 'other' cells.

Measurement of TGF-β1 in BALF: TGF-β1 levels in BALF supernatant were measured using minor modifications of the procedure described by Abe *et al* (1994)[18]. Briefly, BALF supernatant was filtered through two layers of sterile

muslin and pre-spun in Vectaspin micro anopore filters (Whatman International Ltd., Maidstone, Kent) at 13000 rpm for 5 minutes to remove cell debris. The supernatant was then concentrated 5 times by means of Vivaspin 0.5ml concentrators (Vivascience Ltd. Gloucestershire, UK; 10,000 MW cut-off) used according to the manufacturers instructions.

TGF- β 1 is secreted in a latent form therefore acid activation is required to release active TGF- β 1 from the latent complex. Relevant aliquots were acid activated by the addition of 5M HCl. Thereafter the samples were neutralised and submitted for assay.

Mink lung epithelial cells (MLEC) stably transfected with an expression construct containing a truncated plasminogen activator inhibitor-1 (PAI-1) promoter fused to the firefly luciferase gene were used as the reporter system. TGF- β , through its induction of PAI-1 expression, causes dose-dependent increases in luciferase activity in this system [18]. MLECs were maintained at 37°C in Dulbecco's Modified Eagles Medium (DMEM), 10% FBS containing penicillin-streptomycin, L-glutamine and G418 (geneticin 200 μ g/ml) (Life Technologies, Cambridge, UK). The sample assay involved removing and replacing cell medium with 100 μ l BALF supernatant. Assay plates were incubated overnight. Thereafter the cells were washed and lysed and the lysate added to opaque-walled 96-well plates (Dynex Technologies, Middlesex, UK). Samples were read in a Reporter™ microplate luminometer (Turner Designs Inc., Sunnyvale, CA, USA) using a luciferase substrate for a single flash reaction. Samples were read against a standard curve

prepared using appropriate dilutions of human recombinant TGF- β 1 (R&D Systems Europe Ltd, Oxon, UK).

Measurement of BALF immunoglobulin levels: DvA-1 specific IgG levels were measured by standard ELISA. Ninety-six well round-bottom polyvinyl chloride microtitration plates (Dynex Immulon 1B)(Dynex Technologies, Middlesex, UK) were coated with 50 μ l of rDvA-1 antigen (2 μ g/ml in 50mM NaHCO₃ (pH 9.6)) and incubated overnight at 4°C. After washing twice with PBS/0.05% tween20, blocking buffer (2% goat serum in PBS/0.05% tween20) was added to each well and incubated for 30 minutes at 20°C. After washing the plates 3 times 50 μ l of the sample, diluted in PBS/0.05% tween 80, was added. After incubating for 1 hour at 20°C the plates were washed 6 times. 50 μ l mouse anti-sheep IgG monoclonal antibody (VPM6)[19] diluted 1:10 in PBS/0.05% Tween20 was added. After incubation for 1 hour at 20°C the plates were washed 6 times. 50 μ l of the second antibody (biotinylated goat anti-mouse IgG; DAKO, Glostrup, Denmark) diluted in PBS/0.05% Tween20 were added. After incubating for 1 hour at 20°C the plates were washed 6 times. 50 μ l of streptavidin peroxidase diluted 1/2000 in PBS/0.05% Tween20 were added to each well and incubated for 1 hour at 20°C. 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma, Poole, UK) was used as substrate and chromogen for horseradish peroxidase. Colour development was stopped by adding 50 μ l of 0.5M H₂SO₄ to each well.

Plates were read at OD of 450 nm by use of an automated microtitration plate reader (EL311, BioTek Instruments, VT, USA). For a given BALF supernatant sample four dilutions were made. The resultant ODs were multiplied by the

respective dilution factors to give unit measurements and the level of immunoglobulin was taken as the average of these four measurements.

Tissue handling: Lung tissue was fixed by airway instillation of 10% neutral buffered formalin (pH 7.0) at an inflation pressure of 2.5 to 3.0 kPa for 3 days. Following fixation, three tissue blocks were sampled from each lung segment and routinely processed and embedded in paraffin. Serial 4 μ m sections were cut from the tissue blocks and one set stained with toluidine blue. Remaining sections were mounted on polylysine coated slides (BDH Laboratory supplies, Poole, England) for immunohistochemistry.

Immunohistochemistry: Endogenous peroxidase was inactivated by pre-treating sections with periodic acid and sodium borohydride [20]. Sections were treated with trypsin (0.1% trypsin in 0.1% CaCl_2 (pH 7.8)) for 30 min at RT to unmask antigenic sites and thereafter incubated in 4% bovine serum albumin (Sigma, Poole, UK) in PBS for 30 min at RT. For immunohistochemical localisation of SMCP-I, rat mAb anti-SMCP (diluted in 4% BSA/PBS) was added for 1 hr, prior to washing three times in PBS. A biotinylated rabbit anti-rat IgG (diluted in 4% BSA/PBS) was applied for 30 min at RT. The section was washed three times in PBS and Vectastain® ABC reagent (Vector Laboratories, Burlingame, CA, USA) applied for 30 min. After a final wash, peroxidase activity was revealed with 3,3'-diaminobenzidine (DAB). Control sections were stained using a matched rat IgG_{2a} isotype at 10 μ g/ml. For localisation of tryptase, rabbit anti-human tryptase IgG (20 μ g/ml) [12,21], biotinylated goat anti-rabbit antibodies

(Vector Laboratories, Burlingame, CA, USA) and normal rabbit IgG were employed as primary, secondary and source of control antibodies respectively.

Counting technique: Three airways were identified on each section and their location and identity recorded schematically on simple diagrams. The length of the basement membrane of each airway was measured and recorded using a computerised image analysis facility (Quantimet Q500C, Leica, UK). Thereafter the number of stained positive cells in an area of interest defined internally by the airway epithelium and externally by the outer cartilaginous border of the airways of interest were counted and expressed as cells/mm basement membrane. Results from differentially treated segments were collated and the mean values reported.

Experimental design: Two weeks after the last intramuscular injection of DvA-1:ALUM four spatially disparate lung segments were selected on bronchoscopic examination and their position carefully mapped. The segments were randomly assigned to one of two challenge protocols, namely repeated (3x) weekly aerosol challenge with DvA-1 (3xDVA) or repeated (3x) weekly aerosol challenge with saline (3xSAL)(Figure 1). Two weeks after the third challenge, BALF was collected from one segment randomly selected from the pair subjected to each of the challenge protocols. Two months after the third challenge, one of the two segments previously repeatedly challenged with DvA-1 was challenged again with DvA-1 whilst the other was challenged with saline. Similarly, one of the two segments previously repeatedly challenged with saline was challenged again with saline whilst the other was challenged with DvA-1.

Twenty-four hours after the last challenge, sheep were re-anaesthetised and BALF collected from each of the four treated segments (3xSAL:SAL; 3xSAL:DVA; 3xDVA:SAL; 3xDVA:DVA). Three sheep were killed 24 hr after the last challenge to provide tissue for histopathological and immunohistochemical analysis.

Data analysis: Within sheep, between segment comparisons were assessed using Friedman non-parametric tests for related variables. Where significant changes were identified, further analysis was conducted using Wilcoxon signed rank tests to compare between specific segments. Degrees of association between measurements were assessed using the Spearman rank correlation coefficient (r_s).

Results

BALF cytology: Two weeks after the third segmental challenge, BALF cellular parameters did not differ between 3xSAL and 3xDVA segments (data not shown). Twenty four hours after the final segmental challenge, Friedman tests revealed significant differences between segments with respect to total cell counts, comprising changes in the absolute numbers of neutrophils and eosinophils (table 1). BALF from 3xSAL:DVA and 3xDVA:DVA segments contained significantly more cells than 3xSAL:SAL segments ($p<0.05$) with neutrophils the predominant cell type involved ($p<0.01$)(figure 2a). A significant increase in the number of eosinophils in 3xSAL:DVA, 3xDVA:SAL and 3xDVA:DVA segments ($p<0.01$, $p<0.05$ and $p<0.01$ respectively) was noted (figure 2b).

TGF- β 1 levels in BALF: There were no significant differences between segments with respect to levels of total TGF- β 1 in BALF twenty four hours after the final segmental challenge. However, there were significantly increased levels of active TGF- β 1 in the 3xDVA:SAL segments ($p < 0.05$) and a trend towards increased levels in the 3xDVA:DVA segments ($p = 0.08$) relative to levels in the 3xSAL:SAL segments. Levels of total and active TGF- β 1 are depicted in Figure 3. Total TGF- β 1 levels were significantly correlated with absolute numbers of eosinophils in 3xSAL:SAL, 3xSAL:DVA and 3xDVA:DVA segments twenty four hours after the final segmental challenge ($r_s = 0.580$, 0.588 and 0.627 and $p < 0.05$, $p < 0.02$ and $p < 0.02$ respectively). The relationship between absolute numbers of eosinophils and total TGF- β 1 in BALF from 3xDVA:DVA segments is depicted in Figure 4. There were no other significant associations between cellular and immune parameters and levels of total or active TGF- β 1 in BALF twenty four hours after challenge.

BALF Immunoglobulins: Levels of anti-DvA-1 specific IgG in BALF from 3xDVA:DVA and 3xDVA:SAL segments were significantly increased relative to levels in the 3xSAL:SAL segments ($p < 0.01$)(Figure 5).

Histochemical analysis: The number of toluidine blue-stained cells expressed per millimetre airway basement membrane in 3xSAL:DVA, 3xDVA:SAL and 3xDVA:DVA segments did not differ significantly from 3xSAL:SAL segments twenty four hours after the final challenge (Table 2).

Immunohistochemical analysis: Low and highly variable numbers of SMCP-1 immunoreactive cells precluded statistical analysis based on absolute cell

numbers. The numbers of SMCP-1 and tryptase immunoreactive cells were therefore expressed as ratios of the number of toluidine blue-stained cells counted in the same airways. Summary data relating to absolute cell counts is shown in table 2. Similar numbers of tryptase positive and toluidine blue-stained cells were identified in airways from 3xSAL:SAL, 3xSAL:DVA and 3xDVA:SAL segments 24hr after the final challenge (tryptase:toluidine blue ratio 0.93 ± 0.46 , 0.87 ± 0.6 and 1.14 ± 0.96 (mean \pm SD) respectively), however approximately twice as many tryptase positive cells as toluidine blue stained cells were identified in the 3xDVA:DVA segments at this time point (2.04 ± 1.64). Similarly an increased ratio of SMCP-1 positive cells relative to toluidine blue stained cells was identified in airways from 3xDVA:DVA segments at 24hr (SMCP-1:toluidine blue ratio 0.64 ± 0.81) when compared with the same ratio in other segments (0.23 ± 0.33 , 0.26 ± 0.26 and 0.29 ± 0.46 (mean \pm SD) in airways from 3xSAL:SAL, 3xSAL:DVA and 3xDVA:SAL segments respectively) In both instances the increased ratios were significant when compared with ratios in 3xSAL:SAL segments ($p < 0.05$)(Figure 6).

Virtually all cells (>95%) identified by toluidine blue or immunohistochemical staining were located between the epithelial basement membrane and the outer cartilaginous border i.e. intraepithelial cells were rarely identified. Cells were commonly identified surrounding submucosal glands or within and around smooth muscle bundles.

Discussion

Lung nematode infection is characterised by prominent mastocytosis. Crosslinking of pre-bound specific anti-parasite IgE antibody on the surface of these cells is believed to result in degranulation with the release of a variety of mediators which have the collective effect of increasing vascular permeability, causing local vasodilation, bronchial and visceral smooth muscle contraction and local inflammation. Although such effects will contribute towards nematode expulsion, chronic mast cell-mediated inflammation may contribute significantly towards the extensive tissue remodelling that is a feature of lungworm infection in ruminants [1,22]. We sought to develop a model system appropriate to the investigation of the role of mast cells in this regard.

Several factors contributed towards our adoption of a segmental, rather than whole lung, approach to challenge. Wherein responses can be considered local such an approach allows each animal to serve as its own control, thus reducing the extent of inter-animal variability associated with whole lung studies. Responses are limited to defined areas of the lung and as such are associated with no discernible clinical effect. Segmental approaches have hitherto proved valuable in the context of defining, at functional, cellular and immune levels, the local lung response to antigen challenge in both experimental animals and in man [23-30].

Sensitisation with DvA-1 in ALUM resulted in a vigorous systemic antibody response characterised by increased antigen-specific IgG.

Cellular response to antigen challenge was characterised by a neutrophil inflammatory cell infiltrate at 24hr. Although this is consistent with data in dogs in response to particulate [31] and soluble [32] antigen, and in healthy human subjects [33] and subjects with hypersensitivity pneumonitis [34] in response to antigen, some comment is appropriate in relation to the possible influence of endotoxin in contributing to this response. Indeed it has been demonstrated that endotoxin, either sourced to the antigen preparation [35] or arising as a specific consequence of the bronchoscopy procedure [36], may contribute to BAL-induced lung inflammation. A total of 0.8 EU was delivered to each segment during the challenge procedures, an amount considerably less than the 12.5 EU/ml suggested as being more likely to alter the cellular inflammatory response of human subjects to allergen [35]. Although it is acknowledged that species vary considerably in their response to endotoxin [37], in defence of this particular inter-species comparison, it is of note that sheep do appear to share a similar endotoxin sensitivity to man [38]. Thus it is considered unlikely that endotoxin contamination of the antigen preparation made a significant contribution to the observed neutrophilic influx. Further, the adopted segmental approach controls for the possibility of a lung neutrophilic response arising as a consequence of the bronchoscopic procedure itself.

All segments with an immediate or long-term history of exposure to DvA-1 had increased numbers of eosinophils in BALF 24hr after challenge. Although the effect was most striking in segments with concomitant neutrophilic inflammation,

numbers were in addition increased in the 3xDvA:SAL segment relative to the 3xSAL:SAL segment. The median absolute eosinophil count from 3xSAL:SAL segments was 166 cells/ml whereas the median count from 3xDVA:DVA segments was 1.7×10^3 cells/ml, representing 0.2 and 0.8% of the total cell count respectively. By way of comparison, in *Ascaris suum* allergic sheep the whole lung response to inhaled allergen is characterised by less than 0.5% of total cells being identified as eosinophils at the 24hr time point after challenge [39]. Overall, such cell counts are modest both in relation to the eosinophilic response seen during natural lungworm infection [22,40], and in comparison with changes seen in allergic human subjects in response to segmental antigen challenge. In the latter regard, in ragweed-allergic asthmatics, absolute eosinophil counts increased from a mean of 0.49×10^4 /ml BALF (3.7% of total) to 59.0×10^4 /ml (~56%) at 24hr after challenge with antigen [22].

We examined cellular and immune changes at 24h after challenge. In an analogous canine model, the numbers of eosinophils in BALF were significantly raised 4 days after challenge with the relevant antigen [41,42]. Although preliminary data suggests similar eosinophil kinetics in the present model (D. Collie, unpublished observation), further time-course evaluation after antigen challenge will be necessary to fully define such events post-challenge.

The present study demonstrates that antigen specific IgG is present in BALF of segments with a prior history of exposure to a recombinant antigen derived from *D.viviparus*. Indeed the disassociation of antibody levels in BALF from

concomitant inflammation (comparing 3xDVA:DVA and 3xDVA:SAL segments) suggests that antibody is locally derived rather than a reflection of a serum response. These findings are consistent with extensive studies in dogs [43-48].

Our findings demonstrate a significant increase in the ratio of SMCP-1 and tryptase expressing cells, to toluidine blue stained cells in airways from 3xDvA:DvA segments relative to 3xSAL:SAL segments. It is of some interest that the tryptase:toluidine blue ratio was close to 1.0 in all bar the 3xDvA:DvA segment whereas the SMCP:toluidine blue ratio was much lower (~0.25) in these segments. Further immunohistochemical analysis using dual staining is required to ascertain firstly whether these separate proteases are contained within the same population of cells and secondly, whether, as is the case in human bronchi [49], differences exist in relation to protease expression and anatomic sublocation in the airway wall.

Regardless of the specific roles that these proteases play in our model system, clearly there would appear to be a requirement for both inflammatory and local lung immune components in dictating the conditions necessary for their expression. The twin observations that the pleiotropic cytokine, transforming growth factor beta 1 (TGF- β 1), has been shown to play an important role *in vitro* in promoting the expression of mMCP-1 in cultured mBMMC [15] and is raised 24 hr following segmental bronchoprovocation of atopic asthmatics with allergen [33], prompted our measurement of this cytokine in this study.

We were able to demonstrate an increase in the levels of active TGF- β 1 in BALF 24h after challenge in 3xDVA:SAL segments and a trend towards an increase in 3xDVA:DVA segments. The implication would be that lung segments chronically challenged with DvA-1 and which are uniquely characterised by eosinophilia *and* increased levels of local antibody production have in place mechanisms which are responsible for activating TGF- β 1. Principal mechanisms include the cleavage [50] or conformational alteration of latency associated peptide (LAP)[51,52], which noncovalently associates with TGF- β and prevents receptor binding. It remains to be established whether TGF- β 1 is responsible for the increased expression of SMCP-1 in 3xDVA:DVA segments. However, preliminary *in vitro* studies of sheep BMMC suggest that this cytokine does upregulate expression of SMCP-1 (Huntley, JF and Miller HRP, unpublished).

Eosinophils appear to be a primary source of TGF β 1 mRNA [53,54] in asthma, a finding that adds credence to our observed association between the number of eosinophils and the levels of total TGF- β 1 in BALF in all bar the 3xDVA:SAL segment. This latter segment was characterised by a significant increase in active TGF- β 1 and such activation of total TGF- β 1 may have contributed to the observed lack of association with eosinophil numbers in this segment.

In conclusion, we have demonstrated that repeated local lung challenge of systemically sensitised sheep with a recombinant protein derived from the lungworm spp. *Dictoyocaulus viviparus* leads to the development of local

antibody production. Cellular changes 24 hr after the fourth challenge are characterised by an increase in the absolute numbers of neutrophils and eosinophils and are complemented by increased ratios of SMCP-1 expressing cells and tryptase expressing cells to toluidine blue positive cells in airways.

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